Use of an Immunoglobulin G Avidity Assay Based on Recombinant Antigens for Diagnosis of Primary *Toxoplasma gondii* Infection during Pregnancy

Elisa Beghetto,[1] Wilma Buffolano,[2] Andrea Spadoni,[1] Mariassunta Del Pezzo,[3]
Manlio Di Cristina,[1] Olga Minenkova,[1] Eskild Petersen,[4] Franco Felici,[1,5]
and Nicola Gargano[1]*

*Kenton Laboratories, Rome,[1] Department of Pediatrics[2] and Microbiology Laboratories,[3] Federico II University, Naples,
and Department of Microbiological, Genetic and Molecular Sciences, University of Messina, Messina,[5] Italy, and
Department of Gastrointestinal and Parasitic Infections, Statens Serum Institut, Copenhagen, Denmark[4]*

The objective of this work was to develop an antibody-specific immunoglobulin G (IgG) avidity assay to discriminate between acute and latent phases of *Toxoplasma gondii* infection by using recombinant antigens. One hundred twenty-one serum samples from women who developed IgG antibodies against *Toxoplasma* during pregnancy were used. The IgG avidities of antibodies directed against epitopes carried by fragments of GRA3, GRA7, MIC3, and SAG1 antigens were measured by performing parallel enzyme immunoassays. The avidity index for *Toxoplasma*-specific antibodies against a homogeneous mixture of recombinant GRA3, GRA7, MIC3, and SAG1 antigens correlated closely with the IgG avidity of antibodies against lysed whole-cell *T. gondii* antigen. The avidity assay performed with the recombinant MIC3 antigen highlighted the presence of avidity low-antibodies IgG exclusively in sera collected within 2 months after primary infection. The presence of *T. gondii*-specific, low-avidity IgG antibodies against recombinant MIC3 antigen can be used to determine the point of infection with *T. gondii* within a 2-month time frame after infection.

Toxoplasma gondii is an obligate intracellular parasite that is distributed worldwide and responsible for the anthropozoonosis toxoplasmosis. *T. gondii* infection in humans is generally asymptomatic and induces a self-limiting disease. In contrast, primary infection during pregnancy can be transmitted to the fetus throughout the placenta, causing severe disease, such as premature birth, permanent neurological damage, and visual impairment (24). Identifying the gestational age of primary infection is crucial for the clinical management of pregnant women, since the severity of toxoplasmosis for the fetus decreases and transmission rate increases as gestational age progresses (9, 15). Prenatal therapy of gestational toxoplasmosis is generally considered effective for reducing both the incidence of clinical manifestations in infected infants (11) and the vertical transmission rate (8, 26). Thus, prenatal surveillance of *T. gondii* infection in pregnancy is widespread in Europe, enabling the appropriate management of gestational toxoplasmosis as soon as primary infection is diagnosed (5, 11, 27).

Diagnosis of acute infection depends on detection of *Toxoplasma*-specific immunoglobulin G (IgG) and IgM antibodies (1, 24) but does not make it possible to estimate the time of infection with accuracy (12, 17). Measurement of specific IgG avidity has been shown to be the best tool, in combination with IgM analysis, for determining when the infection took place (14, 16, 18, 19, 21, 23, 25). The functional affinity of specific IgG antibodies is initially low after primary antigenic challenge and increases during subsequent weeks and months by antigen-driven B-cell selection. One problem with this method is that some patients have persistent, low-avidity IgG antibodies for many months against lysed whole-cell *Toxoplasma* antigen, emphasizing the need for improvement of the IgG avidity assay (7, 21, 23).

Our strategy was to study the affinity maturation of IgG antibodies against specific *Toxoplasma* recombinant antigens compared to the IgG avidity assay with lysed whole-cell antigen. The IgG avidities of antibodies were analyzed by using sera collected from women for whom the time of infection was known. We show here that a recombinant antigen containing a region of the MIC3 protein represents a promising marker for an avidity assay to improve diagnosis of *T. gondii* infection.

MATERIALS AND METHODS

Serum samples. One hundred twenty-one serum samples from 80 women who were seronegative for *T. gondii* at the beginning of pregnancy but developed IgG antibodies (seroconverted) during gestation were included in the study. The time of infection was defined as the midpoint of the interval of time between the last IgG-negative and the first IgG-positive sample (sampling intervals, usually 4 to 5 weeks).

Sera were collected at different intervals after initial seroconversion as follows: within the 1st month of infection ($n = 11$) and between the 1st and 2nd ($n = 9$), 2nd and 3rd ($n = 9$), 3rd and 4th ($n = 9$), 4th and 5th ($n = 8$), 5th and 6th ($n = 8$), 6th and 12th ($n = 44$), and 12th and 24th ($n = 23$) months of infection.

All samples were collected at the Referral Centre for Perinatal Infections of the Campania Region, Southern Italy (University Federico II of Naples). Sera taken from 30 *Toxoplasma* IgG-negative women referred to the Centre for other infectious diseases (cytomegalovirus, hepatitis C virus, or rubella infections) were used as controls. The serum samples were analyzed in blind fashion.

Clinical management of infected pregnant women. As soon as diagnoses were confirmed, all women were treated up to delivery with an alternate regimen of pyrimethamine-sulfadiazine, folinic acid, and spiramycin.

Recombinant antigens. Four *T. gondii* antigens, GRA3 (amino acids 35 to 133) (4), GRA7 (amino acids 24 to 103) (10), MIC3 (amino acids 233 to 307) (13), and

* Corresponding author. Mailing address: Kenton Laboratories, Via Pontina Km 30.400, 00040 Rome, Italy. Phone: 39-06-91611494. Fax: 39-06-91629012. E-mail: gargano@kenton.it.

SAG1 (amino acids 182 to 303) (6), were used. Recombinant antigens were expressed in bacterial cells as fusion proteins either with glutathione *S*-transferase (GST) or with Lambda protein D (3) and were purified by affinity chromatography as previously described (3). Briefly, recombinant *Escherichia coli* was induced with isopropyl-β-D-thiogalactopyranoside (IPTG), centrifuged, and suspended in STE buffer (10 mM Tris-HCl [pH 8], 150 mM NaCl) containing 100 μg of lysozyme per ml and protease inhibitor cocktail (Boehringer, Mannheim, Germany). The mixture was sonicated at 4°C and Triton X-100 was added to a final concentration of 1%. After centrifugation at $10,000 \times g$ for 30 min at 4°C, the supernatant was recovered and incubated with either glutathione-Sepharose (for GST fusion proteins; Amersham Pharmacia Biotech, Uppsala, Sweden) or Ni-nitrilotriacetic acid resin (D fusion protein; Qiagen). Fusion proteins were eluted by following the manufacturer's instructions and were finally stored at −20°C.

Lysed whole-cell enzyme immunoassays. Lysed whole-cell *T. gondii* antigen was used for determining *Toxoplasma* IgG, IgM, and IgG avidity indexes (Vidas system; bioMeriëux, Marcy l'Etoile, France) and IgA (Platelia Toxo-IgA test; Pasteur, Paris, France).

Recombinant protein enzyme immunoassays. Maxisorb multiwell plates (Nunc, Roskilde, Denmark) were coated with recombinant proteins at a final concentration of 5 μg/ml in coating buffer (50 mM $NaHCO_3$, pH 9.6). After overnight incubation at 4°C, the plates were blocked with 200 μl of blocking solution (5% nonfat dry milk and 0.05% Tween 20 in phosphate-buffered saline [PBS]) per well and subsequently incubated with human sera diluted 1:50 in blocking buffer (100 μl/well). Plates were incubated for 1 h at 37°C and then washed with washing solution (0.05% Tween 20 in PBS). One hundred microliters of alkaline phosphatase-conjugated anti-human IgG antibody (Sigma) diluted 1:7,500 in blocking solution was added to each well. After 30 min at 37°C, the plates were washed and then incubated for 30 min at room temperature with the chromogenic substrate *p*-nitrophenyl phosphate (Sigma) in developing solution (10% diethanolamine [pH 9.8], 0.5 mM $MgCl_2$, 0.05% NaN_3). The results were recorded as the differences between the optical densities (ODs) at 405 nm and at 620 nm, measured with an automated enzyme-linked immunosorbent assay reader (Multiskan Labsystems, Helsinki, Finland).

IgG avidity assay. Maxisorb plates (Nunc) were coated with recombinant proteins as described above. Samples were tested in two fourfold titration rows, starting at a dilution of 1:12.5. After a 1-h incubation at 37°C, plates were washed three times with washing buffer. Row A was then incubated with 200 μl of dissociation buffer (6 M urea and 0.05% Tween 20 in PBS) per well, and row B was incubated with 200 μl of washing buffer per well. After 30 min of incubation at 37°C with vigorous shaking, plates were washed once with washing buffer and processed as described above. Endpoint titers, one for the dissociation buffer and the other for the control, were calculated by using the following formula: endpoint titer = dilution factor × ($OD_{\text{dilution factor}} - OD_{\text{cutoff}}$).

Cutoff values were calculated for each dilution as the means plus 2 standard deviations (SD) of the *Toxoplasma*-negative sample reactivities, with or without urea treatment. Dilution factor values corresponded to the highest dilutions with ODs above the cutoff in assays with and without urea. IgG avidity was calculated as the ratio between the endpoint titer obtained for the reaction including urea and that obtained for the control, expressed as a percentage.

Statistical analysis. Means, medians, ranges, and SD of the avidity measurements for the sera from the different groups are given. Spearman's rank correlation coefficient (r) was used to compare the results obtained with lysed whole-cell *T. gondii* antigen and those obtained with the mixture of recombinant antigens. Results obtained by using single MIC3 antigen were compared by the nonparametric unpaired two-group Mann-Whitney test.

RESULTS

Immunoreactivities of fusion proteins. The immunoreactivities of GST-GRA3, GST-GRA7, GST-MIC3, and D-SAG1 (3) were determined for 60 *Toxoplasma* IgG-positive sera. Thirty *Toxoplasma* IgG-negative sera were assayed as controls. Specific levels of anti-*Toxoplasma* IgG, as determined by the whole-cell *Toxoplasma* immunoassay, ranged from 30 to 3,000 international units (IU)/ml for the positive samples and from 0 to 6 IU/ml for the negative controls (22).

The enzyme-linked immunosorbent assay performance of the four fusion proteins was assessed with the 60 IgG-positive samples, and for each recombinant product the cutoff value

TABLE 1. *Toxoplasma*-specific IgG avidity of 60 serum samples from 49 women with primary infection during gestation

Group or antigen	No. of reactive sera	% Avidity			
		Mean	Median	Range	SD
A[a]					
Whole-cell *Toxoplasma* antigen (Vidas)	13	8.5	6.0	3–28	7.7
Recombinant antigen mix	13	11.6	11.2	6–20	4.8
GST-GRA3	12	9.1	7.9	2–20	5.3
GST-GRA7	12	7.8	6.2	3–17	4.7
GST-MIC3	13	13.0	11.1	3–24	7.4
D-SAG1b	12	12.1	7.8	4–33	10.0
B[b]					
Whole-cell *Toxoplasma* antigen (Vidas)	47	44.3	47.0	2–74	17.4
Recombinant antigen mix	46	44.9	47.2	12–70	13.9
GST-GRA3	39	44.4	41.3	5–91	21.6
GST-GRA7	39	36.5	33.9	5–76	15.8
GST-MIC3	44	63.9	67.4	30–88	16.1
D-SAG1b	43	33.6	31.7	4–90	18.2

[a] Sera were collected within 2 months after acquisition of infection.
[b] Sera were collected after 2 months and before 24 months after acquisition of infection.

was determined as the mean plus 2 SD of the absorbency readings obtained for the 30 *Toxoplasma* IgG-negative sera. D-SAG1b and GST-MIC3 fusion proteins reacted with 92 and 95% of the positive sera, respectively, and both GST-GRA3 and GST-GRA7 reacted with 85% of the positive sera. One of the 30 *Toxoplasma*-negative sera did react with the D-SAG1 antigen and none recognized the GST-GRA3, GST-GRA7, and GST-MIC3 antigens.

The avidity assay with recombinant antigen fragments. The avidities of the immunoglobulins directed against the epitopes carried by the GST-GRA3, GST-GRA7, GST-MIC3, and D-SAG1b fusion proteins were compared with the avidities measured by the lysed whole-cell assay (Vidas; bioMérieux). Serum samples were arbitrarily divided into two groups as follows: sera collected within 2 months of infection and sera collected >2 months after infection. Table 1 shows the IgG avidity indexes for individual recombinant antigens tested as single protein products and as a homogeneous mixture. A similar trend in affinity maturation of antibodies was found when the results obtained with lysed whole-cell *Toxoplasma* antigen and those obtained with a mixture of recombinant antigens were compared ($r = 0.83$; $P < 0.001$; confidence level, 95%). Avidities of antibodies against single antigen fragments were variable, especially for serum samples collected >2 months after infection. In particular, the recombinant MIC3 antigen gave fewer low-avidity results than the other antigens, with a mean avidity of 63.9%. GRA7 and SAG1 recombinant products exhibited the lowest avidities, with respective means of 36.5 and 33.6%. Interestingly, when we used the MIC3 antigen fragment, no overlapping between IgG avidity values for samples collected before and after 2 months of infection was observed.

IgG avidity for single recombinant MIC3 antigen. The clinical usefulness of recombinant antigens in the IgG avidity assay was further investigated by using the GST-MIC3 product. One hundred twenty-one sera were analyzed with the MIC3 anti-

A Vidas IgG-avidity
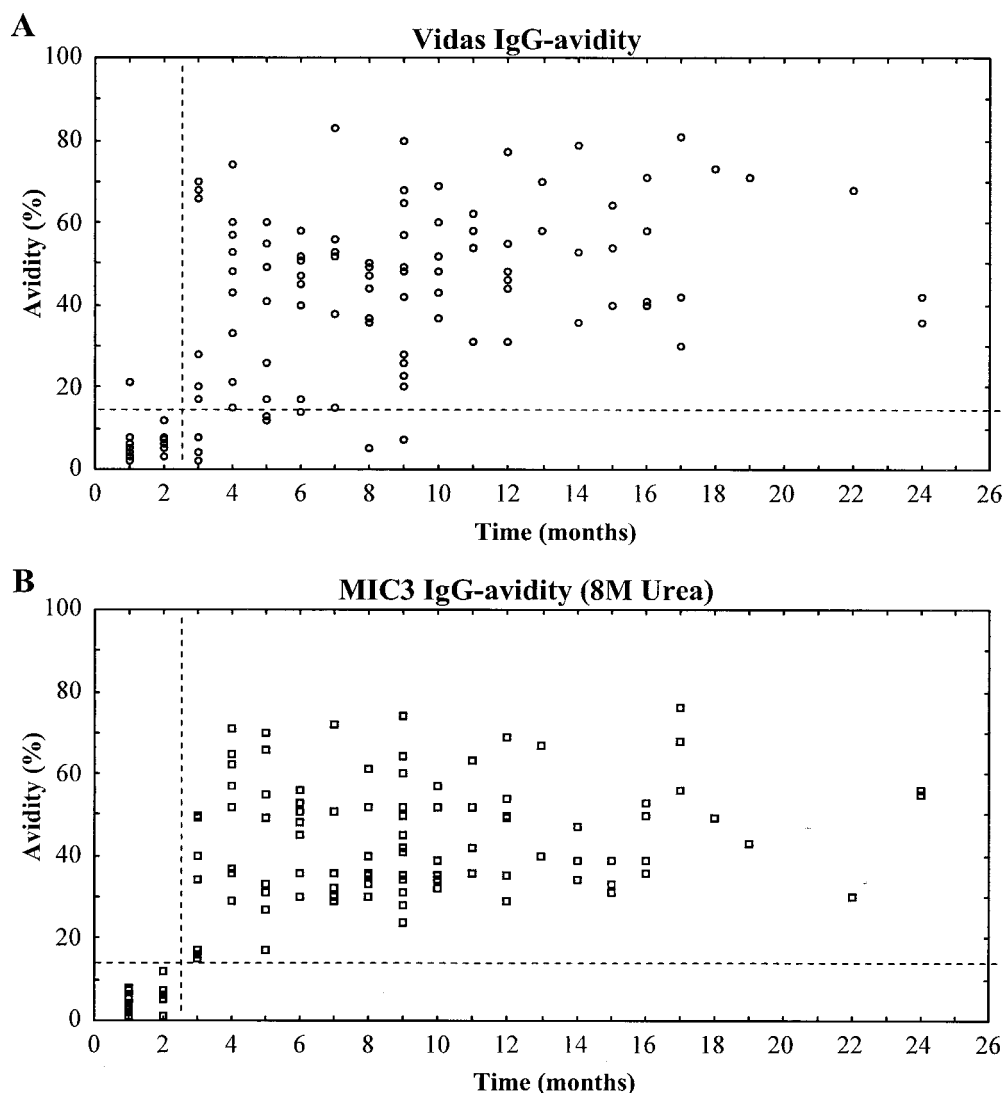

B MIC3 IgG-avidity (8M Urea)

FIG. 1. IgG avidity assay with MIC3 antigen. (A) *Toxoplasma*-specific IgG avidity index for lysed whole-cell *T. gondii* antigen (Vidas system; bioMérieux). (B) *Toxoplasma*-specific IgG avidity index for recombinant GST-MIC3 and elution buffer with 8 M urea.

gen, and the assay was performed with various concentrations of the dissociation agent. As is clearly shown in Fig. 1, when we used the 8 M urea concentration, no overlapping between IgG avidity values of samples collected after 2 and 3 months of infection was observed. The MIC3 IgG avidity index showed no statistical difference between the first and second months after seroconversion ($P = 0.8$) but was significantly different between months 2 and 3 after seroconversion ($P = 0.012$). The MIC3 IgG avidity indexes for months 1 and 2 and for months 3 and 4 after seroconversion were highly significantly different ($P = 0.0002$).

Table 2 shows the results of the MIC3 IgG avidity assay compared to the IgG avidity index obtained with lysed whole-cell antigen. The increase of urea concentration from 6 to 8 M resulted in a marked reduction of avidity index values for both groups, with a mean decrease of 10.3 to 5.3% for group A and 60.9 to 44.4% for group B.

The proportions of sera that were correctly identified as

TABLE 2. *Toxoplasma*-specific IgG avidity of 121 serum samples from 80 women with primary infection during gestation

Group or antigen	n	% Avidity			
		Mean	Median	Range	SD
A[a]					
Whole-cell *Toxoplasma* antigen (Vidas)	20	5.7	4.6	2.3–28	4.2
MIC3 6 M urea	20	10.3	9.3	2.3–23.9	5.6
MIC3 8 M urea	20	5.3	5.3	1.0–12.4	3.0
B[b]					
Whole-cell *Toxoplasma* antigen (Vidas)	101	45.5	46.8	1.8–85.6	19.7
MIC3 6 M urea	97	60.9	61.8	21.6–95.1	16.3
MIC3 8 M urea	97	44.4	42.1	14.9–76.1	14.6

[a] Sera were collected within 2 months after acquisition of infection.
[b] Sera were collected after 2 months and before 24 months after acquisition of infection.

TABLE 3. Proportion of sera correctly identified as having been collected within or after 2 months of primary infection, using either lysed whole-cell *Toxoplasma* antigen (standard assay) or MIC3 avidity assays

Time of serum collection (n)	Diagnostic criterion (% IgG avidity)	% of sera for which collection time was correctly identified	
		Standard assay	MIC3 (8 M urea)
Within 2 mos of infection (20)	<10	90	90
	<14	95	100
	<20	95	100
More than 2 mos after infection (97)	>10	95	100
	<14	92	100
	<20	85	86

having been collected within 2 months or after 2 months of infection by using different diagnostic criteria are shown in Table 3. All serum samples collected >2 months after seroconversion had IgG avidity indexes of >14%, while 92% of the sera analyzed with the whole-cell *Toxoplasma* antigen showed comparable results. This means that the low-avidity result (<14%) obtained with the MIC3 assay might determine, with great accuracy, whether the infection took place within the previous 2 months. In contrast, a high-avidity result (>14%) means that the primary infection occurred >2 months before the test.

Using an avidity ratio cutoff of 14%, the MIC3 IgG avidity index could separate all samples according to infection within 2 months of or >2 months after infection, while the assay using whole-cell *Toxoplasma* antigen showed low avidity for some samples for up to 9 months after infection (Fig. 1). Table 4 shows results for individual patients, and it is noteworthy that all these sera had specific IgM antibodies, even many months after primary infection.

DISCUSSION

Most assays for *T. gondii* IgG avidity routinely detect antibodies against whole tachyzoite cell extracts. Consequently, the avidity index is a measure of polyclonal IgG avidity that includes the contribution of antibody avidity against single anti-

gens. Our starting hypothesis was that affinity maturation of antibodies against single epitopes represented by recombinant *Toxoplasma* antigens might follow a different pattern. Few studies have investigated this hypothesis (20, 28), and little is known about the use of single antigens in the avidity assay.

In a previous study, we identified and characterized a panel of immunodominant regions of *T. gondii* proteins (2, 3). In this study, we investigated further the IgG avidities of specific *Toxoplasma* antibodies directed against four distinct antigenic regions. There was a close correlation between the avidity indexes for lysed whole-cell *Toxoplasma* antigen and those for a recombinant antigen mixture. However, the assays performed with whole parasite extract and with a recombinant antigen mixture detected low-avidity antibodies in the sera of patients who seroconverted >2 months before sampling, which is in accordance with other studies which have found that low-avidity IgG antibodies may persist for many months beyond the acute infection (7, 21, 23). Therefore, the use of this mixture of recombinant antigens would have the same limitation as tests based on whole-cell lysate antigen.

However, by using the GST-MIC3 antigen, we could detect low-avidity IgG antibodies exclusively in serum samples from pregnant women infected within 2 months prior to the test. The presence of low-avidity antibodies against the GST-MIC3 antigen therefore reduces the time frame in which the acute *T. gondii* infection may have occurred to a 2-month window. The whole-cell lysate assay detected low-avidity antibodies in sera from patients who had seroconverted up to 9 months previously, in practice making a low-avidity index of almost no use for the clinician in the timing of infection. Previous studies have also found that low-avidity IgG antibodies may persist for many months beyond the acute infection (7, 21, 23).

The MIC3 antigen fragment was found to be a good marker for diagnosing recently acquired infections, and in contrast to the lysed whole-cell assay, both high- and low-avidity results may be used to determine the time of infection. This is important to know when pregnant women ask for a test of immunity, which in most countries, including Italy, is usually performed between gestational weeks 8 and 14.

In conclusion, our results show that the avidity assay based on recombinant antigens has potential clinical usefulness for diagnosing the acute phase of *T. gondii* infection during preg-

TABLE 4. Comparison of whole-cell *Toxoplasma* antigen (standard assay) and MIC3 avidity determinations for serum samples from patients in which the lysed whole-cell assay inaccurately estimated the time of infection

Patient no.	Time after infection (mos)	IgG concn (IU/ml) (cutoff = 10)	IgM concn (IU/ml) (cutoff = 0.65)	Lysed whole-cell assay		MIC3 assay (8 M urea)	
				% Avidity	Interpretation[a]	% Avidity	Interpretation[a]
T20	1	83	1.0	20.7	H	7.8	L
T94	3	1,152	0.79	8.3	L	14.9	H
T33	3	300	0.67	3.9	L	17.0	H
T58	3	300	2.34	1.8	L	34.0	H
T87	5	60	1.17	12.0	L	33.2	H
T46	5	175	1.04	12.8	L	31.2	H
T55	6	870	0.92	13.9	L	35.8	H
T37	8	78	1.41	5.3	L	51.5	H
T54	9	137	2.43	6.5	L	28.3	H

[a] Diagnostic criteria were as follows: if avidity was <14% (L, low avidity), infection was acquired within 2 months previous; if avidity was >14% (H, high avidity), infection was acquired more than 2 months previous.

nancy. It will be interesting to investigate further the presence of other antigens that might be useful, in combination with the MIC3 fragment, to further enhance the power of the IgG avidity assay for the diagnosis of *T. gondii* infection during pregnancy.

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